The neuroprotective properties of bis(7)-tacrine, a novel dimeric acetylcholinesterase (AChE) inhibitor, on glutamate-induced excitotoxicity were investigated in primary cultured cerebellar granule neurons (CGNs). Exposure of CGNs to 75 μM glutamate resulted in neuronal apoptosis as demonstrated by Hoechst staining, TUNEL, and DNA fragmentation assays. The bis(7)-tacrine treatment (0.01–1 μM) on CGNs markedly reduced glutamate-induced apoptosis in dose- and time-dependent manners. However, donepezil and other AChE inhibitors, even at concentrations of inhibiting AChE to the similar extents as 1 μM bis(7)-tacrine, failed to prevent glutamate-induced excitotoxicity in CGNs; moreover, both atropine and dihydro-β-erythroidine, the cholinergic receptor antagonists, did not affect the anti-apoptotic properties of bis(7)-tacrine, suggesting that the neuroprotection of bis(7)-tacrine appears to be independent of inhibiting AChE and cholinergic transmission. In addition, ERK1/2 and p38 pathways, downstream signals of N-methyl-D-aspartate (NMDA) receptors, were rapidly activated after the exposure of glutamate to CGNs. Bis(7)-tacrine inhibited the apoptosis and the activation of these two signals with the same efficacy as the co-application of PD98059 and SB203580. Furthermore, using fluorescence Ca2⁺ imaging, patch clamp, and receptor-ligand binding techniques, bis(7)-tacrine was found to effectively buffer the intracellular Ca2⁺ increase triggered by glutamate, to reduce NMDA-activated currents and to compete with [3H]MK-801 with an IC50 value of 0.763 μM in rat cerebellar cortex membranes. These findings strongly suggest that bis(7)-tacrine prevents glutamate-induced neuronal apoptosis through directly blocking NMDA receptors at the MK-801-binding site, which offers a new and clinically significant modality as to how the agent exerts neuroprotective effects.

* The neuroprotective properties of bis(7)-tacrine, a novel dimeric acetylcholinesterase (AChE) inhibitor, on glutamate-induced excitotoxicity were investigated in primary cultured cerebellar granule neurons (CGNs). Exposure of CGNs to 75 μM glutamate resulted in neuronal apoptosis as demonstrated by Hoechst staining, TUNEL, and DNA fragmentation assays. The bis(7)-tacrine treatment (0.01–1 μM) on CGNs markedly reduced glutamate-induced apoptosis in dose- and time-dependent manners. However, donepezil and other AChE inhibitors, even at concentrations of inhibiting AChE to the similar extents as 1 μM bis(7)-tacrine, failed to prevent glutamate-induced excitotoxicity in CGNs; moreover, both atropine and dihydro-β-erythroidine, the cholinergic receptor antagonists, did not affect the anti-apoptotic properties of bis(7)-tacrine, suggesting that the neuroprotection of bis(7)-tacrine appears to be independent of inhibiting AChE and cholinergic transmission. In addition, ERK1/2 and p38 pathways, downstream signals of N-methyl-D-aspartate (NMDA) receptors, were rapidly activated after the exposure of glutamate to CGNs. Bis(7)-tacrine inhibited the apoptosis and the activation of these two signals with the same efficacy as the co-application of PD98059 and SB203580. Furthermore, using fluorescence Ca2⁺ imaging, patch clamp, and receptor-ligand binding techniques, bis(7)-tacrine was found to effectively buffer the intracellular Ca2⁺ increase triggered by glutamate, to reduce NMDA-activated currents and to compete with [3H]MK-801 with an IC50 value of 0.763 μM in rat cerebellar cortex membranes. These findings strongly suggest that bis(7)-tacrine prevents glutamate-induced neuronal apoptosis through directly blocking NMDA receptors at the MK-801-binding site, which offers a new and clinically significant modality as to how the agent exerts neuroprotective effects.

** To whom correspondence should be addressed: Dept. of Biochemistry, Hong Kong University of Science & Technology, Clear Water Bay, Kowloon, Hong Kong. Tel.: 852-2358-7293; Fax: 852-2358-1552; E-mail: bcyyfhan@ust.hk.

*** From the Departments of Biochemistry and Biobiology, Hong Kong University of Science and Technology, Kowloon, Hong Kong, the Department of Molecular and Cellular Neurobiology, Tongji Medical College of Huazhong University of Science and Technology, Wuhan 430030, China, the Laboratory of Molecular and Cellular Neurobiology, National Institute on Alcohol Abuse and Alcoholism, Bethesda, Maryland 20892, the Department of Chemistry, Virginia Tech, Blacksburg, Virginia 24061, the Mayo Foundation for Medical Education and Research, Rochester, Minnesota 55905, and the Department of Pharmacology, Northern Campus of Sun Yat-sen University, Guangzhou 510080, China.

The abbreviations used are: AChE, acetylcholinesterase; AD, Alzheimer disease; CGN, cerebellar granule neuron; DIV, days in vitro; E2020, donepezil; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; MKK, mitogen-activated protein kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; NMDA, N-methyl-D-aspartate; FDA, fluorescein diacetate; ANOVA, analysis of variance; APV, 2-amino-5-phosphonovalerate; DNQX, 6,7-dinitroquinoxaline-2,3-dione; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione.

 Novel Dimeric Acetylcholinesterase Inhibitor Bis(7)-tacrine, but Not Donepezil, Prevents Glutamate-induced Neuronal Apoptosis by Blocking N-Methyl-D-aspartate Receptors

Received for publication, September 27, 2004, and in revised form, February 3, 2005
Published, JBC Papers in Press, February 14, 2005, DOI 10.1074/jbc.M411085200
rons and a subsequent neuronal death (18–20). Several kinase pathways, mainly including extracellular signal regulated-kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways, have been demonstrated to be involved in glutamate-induced apoptosis and to be responsible for the downstream signals of overloading intracellular Ca$^{2+}$ (21, 22). The core units (Raf, MEK1/2, and ERK1/2) of ERK pathway signaling cascade are in turn activated by a wide variety of receptors involved in growth and differentiation including receptor tyrosine kinases, integrins, and ion channels. An activated ERK dimer can regulate targets in the cytosol and also translocate to the nucleus where it phosphorylates a variety of transcription factors regulating gene expression (23). In neurons, glutamate overactivates NMDA receptors leading to the overloading of intracellular calcium and the activation of the ERK pathway that mediates apoptosis (21). Mitogen-activated protein kinase kinases 3/6 (MKK3/6) have high activating toward p38 MAPK, which can directly phosphorylate and regulate several transcription factors such as activating ability transcription factor 2 (24).

Several studies suggest that p38 pathway is involved in glutamate-induced apoptosis in neurons in vitro and in vivo (22, 25). However, the relationship between ERK and p38 pathways in glutamate-induced apoptosis has not yet been delineated.

The current studies were undertaken to evaluate the ability of bis(7)-tacrine to prevent glutamate-induced apoptosis in CGNs and then to systematically delineate whether this agent affected the key steps of glutamate-induced apoptosis from ERK and p38 pathways to intracellular Ca$^{2+}$ to NMDA receptors. It appears that bis(7)-tacrine prevents glutamate-induced apoptosis in neurons by directly blocking NMDA receptors at the MK-801-binding site yet independent of the inhibition on AChE and cholinergic transmission.

**EXPERIMENTAL PROCEDURES**

**Primary Cerbellar Granule Neurons Cultures**—Rat cerebellar granule neurons were prepared from 8-day-old Sprague-Dawley rat (The Animal Care Facility, Hong Kong University of Science & Technology) as described previously (26). Briefly, neurons were seeded at a density of 2.0 $\times$ 10^5 cells/ml in basal modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine, and penicillin (100 units/ml)/streptomycin (100 $\mu$g/ml). Cytosine arabinoside (10 $\mu$M) was added to the culture medium 24 h after plating to limit the growth of non-neuronal cells. With the use of this protocol, 95–99% of the cultured cells were granule neurons. The experiments were performed after 8 days in culture.

**Measurement of Neurotoxicity**—The percentage of surviving neurons in the presence of bis(7)-tacrine and/or glutamate was estimated by determining the activity of mitochondrial dehydrogenases with 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (26). The assay was performed according to the specifications of the manufacturer (MTT kit I; Roche Applied Science). Briefly, the neurons were cultured in 96-well plates, 10 $\mu$l of 5 mg/ml MTT labeling reagent was added to each well containing cells in 100 $\mu$l of medium, and the plate was incubated for 4 h in a humidified incubator at 37 °C. After the incubation, 100 $\mu$l of the solvating solution (0.01N HCl in 10% SDS solution) was added to each well for 17–18 h. The absorbance of the samples was measured at a wavelength of 570 nm with 630 nm as a reference wavelength. Unless otherwise indicated, the extent of MTT conversion in cells was measured by MTT assay. All of the data, expressed as percentages of control, were the means ± S.E. of three separate experiments. *, $p < 0.05$; **, $p < 0.01$ versus the corresponding glutamate groups in A and C or versus control at the same time in B + +, $p < 0.01$ versus glutamate group at the same time in B (ANOVA and Dunnett’s test).

**FDA Staining Assay**—Viable granule neurons were stained with fluorescein formed from FDA, which is de-esterified only by living cells (27). Briefly, after incubation with FDA (10 $\mu$g/ml) for 10 min, the neurons were examined and were photographed using UV light microscopy at 520 nm, compared with pictures photographed under phase contrast microscopy.

**Hoechst Staining Assay**—Chromatin condensation was detected by nucleus staining with Hoechst 33342 as previously described (26). CGNs (2.5 $\times$ 10^5 cells) grown in a 100-mm dish were lysed in 10 mM Tris-HCl (pH 7.5) containing 10 mM EDTA and 0.2% Triton X-100. The lysate was cen-

**FIG. 1. Glutamate induces a dose- and time-dependent cell death blocked by bis(7)-tacrine.** A, at 8 DIV, CGNs were exposed to 1 $\mu$M bis(7)-tacrine for 2 h before the addition of glutamate (Glu) at the different concentrations indicated. Cell viability was measured at 24 h after glutamate. B, compared with normal neurons, CGNs were preincubated with or without 1 $\mu$M bis(7)-tacrine for 2 h and then exposed to 75 $\mu$M glutamate at different times indicated. Cell viability was measured at the indicated times. C, CGNs were preincubated with bis(7)-tacrine at different concentrations indicated and were exposed to 75 $\mu$M glutamate 2 h later. At 24 h after glutamate challenge, cell viability was measured by MTT assay. All of the data, expressed as percentages of control, were the means ± S.E. of three separate experiments. *, $p < 0.05$; **, $p < 0.01$ versus the corresponding glutamate groups in A and C or versus control at the same time in B + +, $p < 0.01$ versus glutamate group at the same time in B (ANOVA and Dunnett’s test).

**DNA Fragmentation Assay**—DNA fragmentation was assessed using a soluble DNA preparation as previously described (26). CGNs (2 $\times$ 10^5 cells) grown in a 100-mm dish were lysed in 10 mM Tris-HCl (pH 7.5) containing 10 mM EDTA and 0.2% Triton X-100. The lysate was cen-
trifuged at 12,000 × g for 10 min. The supernatant was treated with proteinase K (0.3 mg/ml) and RNase A (0.3 mg/ml) and then extracted in the presence of 300 mM NaAc. The DNA was precipitated with isopropanol and dissolved in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. The DNA was electrophoresed in 1.5% agarose gel in Tris borate-EDTA buffer. The DNA bands were then imaged by ethidium bromide staining and photographed.

**TUNEL Assay**—CGNs were plated onto 25-mm-round, 1-mm-thick glass coverslips (Fisher) precoated with poly-L-lysine (1%). According to the protocol of TUNEL (TB235; Promega, Madison, WI), the neurons were washed with PBS, fixed with 4% paraformaldehyde for 25 min, and rinsed three times with PBS. The cells were then permeabilized with 0.2% Triton X-100 in PBS and then rinsed twice with PBS and equilibrated with 100 µl of equilibration buffer at room temperature for 10 min. The DNA nick labeling reaction was performed using 50 µl of deoxynucleotidyltransferase incubation buffer, including 45 µl of equilibration buffer, 5 µl of nucleotide mix, and 1 µl of deoxynucleotidyltransferase enzyme for 60 min at 37 °C, and then the reaction was terminated by immersing the slides in 2× SSC for 15 min at room temperature. The samples were then rinsed three times with PBS and mounted for analyzing under a fluorescence microscope using a standard fluorescence filter set to view the microscope using a standard fluorescence enhancement system and exposed to Kodak autoradiographic films.

**AChE Activity Assay**—AChE activity was determined by using a modified Ellman’s assay (2). Briefly, the supernatants were collected from cell lysates (buffer: 20 mM Tris-HCl, pH 8.0, 0.1% Triton X-100) of CGNs 4 h after glutamate challenge by centrifugation at 14,000 × g for 5 min. Each supernatant at 30 µl for the following reaction was first incubated with 0.1 mM tetraisopropylphosphoramide (Sigma) for 10 min to inhibit the butyrylcholinesterase activity. By using acetylthiocholine iodide at 0.625 mM as AChE substrate and 5,5'-dithiobis-(2-nitrobenzoic acid) at 0.5 mM as the color indicator, hydrolysis reaction at 37 °C was monitored by measuring optical density at 405 nm at 30-min intervals for 2 h. Whole protein concentrations were determined by the BCA assay (Pierce) (26), and the specific activity of AChE was calculated by the data from three independent experiments.

**Western Blot Assay**—Western blot analysis was performed as described previously (26). Briefly, the neurons were lysed by adding SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% (w/v) bromphenol blue). The samples were resolved by SDS-PAGE with the use of 12% acrylamide gels, as indicated in the legends. The proteins were transferred to Hybond-P membranes (polyvinylidene difluoride). The membranes were incubated with primary antibodies (1:1000). After incubation with the primary antibodies, the filters were washed and subsequently were developed with an enhanced chemiluminescence system and exposed to Kodak autoradiographic films.

**Kinase Assay**—p38 kinase activity was measured using immune complex kinase by the nonradioactive immunoprecipitation kinase kit (9820; Cell Signaling, Beverly, MA) (26). In brief, CGNs were washed with cold PBS and extracted in an ice-cold lysis buffer. For the p38 kinase assay, cell lysates from CGNs with different treatments were incubated overnight with the phosphorylated p38 antibody immobilized by cross-linking to agarose hydrazide beads. The beads were washed twice with a lysis buffer and twice with a kinase buffer. The kinase reaction was started by adding 2 µg of activating transcription factor 2

FIG. 2. Bis(7)-tacrine blocks the hallmarks of apoptosis induced by glutamate in CGNs. A, at 5 DIV, CGNs were preincubated with or without 1 µM bis(7)-tacrine or 50 µM E2020 and exposed to 75 µM glutamate (Glu) 2 h later. At 24 h after glutamate challenge, CGNs were assayed with a phase contrast microscope, FDA staining, Hoechst 33324 staining, or TUNEL. B, the counts of apoptotic bodies by Hoechst staining as in A. The data, expressed as the percentages of glutamate, were the means ± S.E. of three separate experiments; **, p < 0.01 versus control (ANOVA and Dunnett’s test). C, CGNs were preincubated with or without 1 µM bis(7)-tacrine or 50 µM E2020 and exposed to 75 µM glutamate (Glu) 2 h later. At 24 h after glutamate challenge, the DNA fragmentations were extracted from the CGNs, and then agarose gel electrophoresis and ethidium bromide staining were used to visualize DNA extracted from the above samples. The positive control of DNA fragmentations was from low potassium-induced (5 mM KCl 2 h later).
fusion protein (19–96) as the substrate of p38 and 200 μM ATP and terminated by adding 3 mM SDS sample buffer after incubation for 30 min at 30 °C. In some experiments, bis(7)-tacrine or SB203580 was added 30 min prior to the start of reaction. The samples were analyzed using Western blot assay with the phospho-activating transcription factor 2 antibody.

Confocal Laser Scanning Microscopy—A confocal laser scanning microscope was used to evaluate relative changes in intracellular calcium concentrations ([Ca^{2+}]_i) by monitoring Fluo-3 fluorescence after intracellular cleavage of superfused Fluo-3 acetoxymethylester (5×10^{-6} M, with excitation at 488 nm and emission at >510 nm) (26). In brief, the neurons were stained with 5×10^{-6} M Fluo-3 acetoxymethylester for 30 min in 37 °C incubator and then washed three times with a balanced salt solution containing 154 mM NaCl, 25 mM KCl, 0.035 mM Na_2HPO_4, 2.3 mM CaCl_2, 3.6 mM NaHCO_3, 5.6 mM glucose, 5 mM HEPES, pH 7.4. The fluorescence images were analyzed using the MetaMorph software package (Universal Imaging Co., Downingtown, PA). The data were obtained by evaluating the fluorescence (F) from selected areas within a cell, following subtraction of background fluorescence, and division by the fluorescence intensity before drug application (F_0), expressed as F/F_0. Confocal images were taken and stored every 5–10 s. Bis(7)-tacrine was added to the balanced salt solution 30 min prior to glutamate.

Whole Cell Electrophysiological Analysis—Cultures of hippocampal neurons on glial feeder layers were prepared from 15–17-day fetal mice for the whole cell patch clamp analysis (28). Hippocampi were dissected in Hanks' buffered salt solution containing 10 mM HEPES, DNase type I (Roche Applied Science), and 1 mM sodium pyruvate (pH 7.4); incubated in 0.25% trypsin (Invitrogen) in Hanks' buffered salt solution at 37 °C for 15 min; washed three times in Hanks' buffered salt solution at room temperature; and triturated 30–40 times using a fire-polished Pasteur pipette. The neurons were plated on confluent layers of hippocampal glia in minimum essential medium containing 10% heat-inactivated equine serum (HyClone, Logan, UT) and 1 mM sodium pyruvate. After 4 h, half of this medium was replaced with a maintenance medium consisting of minimum essential medium, 1 mM sodium pyruvate, and N2 serum supplement (Invitrogen); this medium was subsequently given half-changes weekly. The neurons were cultured for 2 weeks prior to use in experiments.

Whole cell patch clamp recording was carried out at room temperature using an EPC-7 patch clamp amplifier (28). Membrane potential was held at -60 mV. The data were filtered at 2 kHz (8-pole Bessel) and acquired on a computer (5-kHz sampling frequency) during experiments using a DigiData 1200A interface and pCLAMP software (Axon Instruments, Inc., Union City, CA). The neurons were superfused at 1–2 ml min^{-1} in an extracellular medium containing 150 mM NaCl, 5

![Image](image-url)
Bis(7)-tacrine Prevents Neuronal Apoptosis

RESULTS

Bis(7)-tacrine Prevents Glutamate-induced Cell Death in CGNs—Prior to investigating the effects of bis(7)-tacrine on glutamate excitotoxicity, a dose- and time-dependent excitotoxicity model in CGNs was firstly established. At 8 days in vitro (DIV), CGNs were exposed continuously to 25–300 μM glutamate for 24 h or 75 μM glutamate alone at different times. Cell viability was measured by MTT assay. Glutamate at 75 μM induced a time-dependent neuronal death starting at 12 h and culminating at 48 h (Fig. 1A). CGNs were pretreated with the gradually increasing concentrations of bis(7)-tacrine for 2 h and exposed to 75 μM glutamate for 24 h or indicated times or pretreated with 1 μM bis(7)-tacrine for 2 h and exposed to different concentrations of glutamate for 24 h. It was found that bis(7)-tacrine prevented 75 μM glutamate-induced cell death in CGNs in a dose-dependent fashion (Fig. 1C). Bis(7)-tacrine at 1 μM retained its neuroprotective effects for at least 60 h after glutamate challenge (Fig. 1B). With the increase of glutamate concentrations, the neuroprotective properties of bis(7)-tacrine gradually decreased and were completely lost when CGNs were exposed to over 300 μM glutamate (Fig. 1A).

Bis(7)-tacrine Inhibits Glutamate-induced Apoptosis in CGNs—In CGNs, glutamate causes necrosis and/or apoptosis depending on the experimental conditions used, and at low concentrations, glutamate more easily induces apoptosis but
diisopropylfluorophosphate at 10 μM and harvested at different times after glutamate challenge. Phospho-MKK3/6 and phospho-p38 were detected by Western blot. Bis(7)-tacrine dose-dependently inhibited the activation of p38 caused by glutamate. Left panel, CGNs were pretreated with SB 203580 at 5 μM or bis(7)-tacrine at different concentrations for 2 h and then exposed to 75 μM glutamate for 1 h. p38 kinase activity was assayed using immune complex kinase by the nonradioactive immunoprecipitation kinase kit as described under “Experimental Procedures.” Right panel, phospho-p38 was extracted from CGNs exposed to 75 μM glutamate for 1 h. SB 203580 at 5 μM or bis(7)-tacrine at the indicated concentrations were added to aliquots of the kinase at 30 min prior to the start of the kinase reaction as described under “Experimental Procedures.”

Bis(7)-tacrine prevents the activation of p38 caused by glutamate in CGNs. A, bis(7)-tacrine time-dependently prevented glutamate-evoked increase of phospho-MKK3/6 and phospho-p38 MAPK. CGNs were exposed to 75 μM glutamate (Glu) in the presence or the absence of Bis(7)-tacrine at 1 μM and harvested at different times after glutamate challenge. Phospho-MKK3/6 and phospho-p38 were detected by Western blot. B, bis(7)-tacrine dose-dependently inhibited the activation of p38 caused by glutamate. Left panel, CGNs were pretreated with SB 203580 at 5 μM or bis(7)-tacrine at different concentrations for 2 h and then exposed to 75 μM glutamate for 1 h. p38 kinase activity was assayed using immune complex kinase by the nonradioactive immunoprecipitation kinase kit as described under “Experimental Procedures.” Right panel, phospho-p38 was extracted from CGNs exposed to 75 μM glutamate for 1 h. SB 203580 at 5 μM or bis(7)-tacrine at the indicated concentrations were added to aliquots of the kinase at 30 min prior to the start of the kinase reaction as described under “Experimental Procedures.”

not necrosis (30). The assays by Hoechst 33342 staining and TUNEL demonstrated that 75 μM glutamate-induced apoptosis and bis(7)-tacrine could prevent glutamate-induced apoptosis in our system. At 8 DIV, CGNs were pretreated with 1 μM bis(7)-tacrine and exposed to 75 μM glutamate 2 h later, and the phase contrast microscopy and FDA staining assays showed that bis(7)-tacrine significantly blocked the loss of neurons and reversed the morphological alteration, including unhealthy bodies and broken extensive neuritic network, induced by glutamate (Fig. 2A). The Hoechst 33342 staining assay showed that bis(7)-tacrine significantly reversed nuclear condensation, whereas the TUNEL assay proved that bis(7)-tacrine blocked glutamate-induced increase in TUNEL-positive neurons (Fig. 2A). The counts of apoptotic bodies stained by Hoechst 33342 showed that bis(7)-tacrine significantly blocked apoptotic bodies by 80% induced by glutamate (Fig. 2B). To further confirm neuronal apoptosis caused by glutamate, a DNA fragmentation gel assay was conducted on the above mentioned sister CGNs cultures. The results showed that bis(7)-tacrine substantially blocked the DNA fragmentation (DNA “ladder”) in CGNs induced by glutamate (Fig. 2C).

The Neuroprotective Effects of Bis(7)-tacrine Are Independent of AChE Inhibition and Cholinergic Transmission—Bis(7)-tacrine is a novel, potential, and selective inhibitor of AChE, and it is unclear whether the neuroprotective properties of bis(7)-tacrine were produced by inhibiting AChE activity. Therefore, bis(7)-tacrine, together with two other reversible AChE inhibitors (tacrine and E2020) and one irreversible AChE inhibitor (diisopropylfluorophosphate), was used to investigate whether they could prevent glutamate-induced apoptosis in CGNs. With MTT assay, it was shown that tacrine or E2020 at 1–50 μM, and diisopropylfluorophosphate at 10 μM failed to efficiently block apoptosis in CGNs induced by 75 μM glutamate (Fig. 3A and B). Although 50 μM of E2020 and 1 μM of bis(7)-tacrine possessed a similar effect on inhibiting the activity of AChE (Fig. 3C), E2020 failed to block the neuronal loss, DNA condensation, TUNEL-positive neurons, and DNA fragmentations caused by 75 μM glutamate in CGNs (Fig. 2).

Activation of cholinergic receptors is able to prevent neuronal apoptosis (27, 31). Bis(7)-tacrine inhibits the activity of AChE and indirectly improves the increase of concentrations of acetylcholine in vitro or in vivo, which may contribute to the therapeutic effects against AD (2, 3). To investigate whether the neuroprotective properties of bis(7)-tacrine was produced by activating either subtype of cholinergic receptors directly and/or indirectly, both atropine (a muscarinic receptor antagonist) and dihydro-β-erythroidine (a nicotinic cholinergic receptor antagonist) were used. By the same cultured conditions mentioned above, it was shown that 10 μM atropine, 50 μM dihydro-β-erythroidine, or their combination failed to antagonize the neuroprotective properties of 1 μM bis(7)-tacrine against 75 μM glutamate-induced apoptosis in CGNs (Fig. 3D).

Bis(7)-tacrine Inhibits the Activations of ERK and p38 Pathways Caused by Glutamate—Both ERK and p38 pathways are associated with glutamate-induced apoptosis in neurons (21, 22). To determine whether ERK and p38 pathways are involved in glutamate-induced apoptosis in our cultured CGNs, the specific inhibitors of MEK1/2 (U0126) and the specific inhibitors of p38 (SB 203580) were selected to pretreat the CGNs for 2 h before adding glutamate. It was observed that U0126 or SB203580 partially prevented glutamate-induced apoptosis in CGNs at 5 μM or bis(7)-tacrine at the indicated concentrations were added to aliquots of the kinase at 30 min prior to the start of the kinase reaction as described under “Experimental Procedures.”

To examine whether bis(7)-tacrine protected neurons through inhibition of ERK pathway, the levels of phospho-Raf, phospho-MEK1/2 and phospho-ERK1/2 were assayed by immunoblots. As shown in Fig. 5A, glutamate at 75 μM caused the increase of phospho-ERK1/2, and bis(7)-tacrine inhibited this event in a time-dependent manner. On the other hand, glutamate also caused the rise of phospho-Raf, phospho-MEK1/2, and phospho-ERK1/2 in a time-dependent manner, which peaked at 15–30 min (Fig. 5B) and returned to their normal levels at 6, 10, and 12 h after glutamate challenge, respectively.
Bis(7)-tacrine significantly inhibited the phosphorylation levels of these three kinases increased by glutamate at 30 min, whereas U0126 only inhibited the rise in phospho-ERK1/2 (Fig. 5C).

To further investigate whether bis(7)-tacrine prevented glutamate-induced apoptosis by inhibition of p38 pathway, the level of phospho-p38 and the activity of p38 kinase were assayed by immunoblots. It was found that 75 μM glutamate caused the increase of phospho-MKK3/6 and phospho-p38 in a time-dependent manner, and 1 μM bis(7)-tacrine reversed the increase of phospho-MKK3/6 and phospho-p38 caused by glutamate (Fig. 6A). Using the kinase assay both in vivo and in vitro as described under “Experimental Procedures,” it was demonstrated that bis(7)-tacrine inhibited the activation of p38 caused by 75 μM glutamate in a dose-dependent manner in vivo, but did not directly inhibit the activity of phospho-p38 in vitro using activating transcription factor 2 as a substrate (Fig. 6B).

**Bis(7)-tacrine Reduces the Glutamate-evoked Increase of Intracellular Ca^{2+}**—According to the above results, bis(7)-tacrine can inhibit the activation of ERK and p38 pathways caused by glutamate, indicating that bis(7)-tacrine may inhibit the common upstream modules of ERK and p38 pathways. Glutamate can induce the overloading of intracellular Ca^{2+} in CGNs, and
the intracellular Ca\(^{2+}\) at high concentrations is able to activate ERK and p38 pathways (21, 22). To investigate whether bis(7)-tacrine could interfere with intracellular calcium release, Fluo-3 fluorescence was used for determining the Ca\(^{2+}\) levels in CGNs. It could be observed that 75 μM glutamate caused a persistent intracellular Ca\(^{2+}\) increase (Fig. 7, A and B). In treatment groups, the neurons were pretreated with a vehicle, 1 μM MK-801, or 1 μM bis(7)-tacrine for 2 h and then exposed to 75 μM glutamate. The intracellular Ca\(^{2+}\) was measured during these times. Compared with MK-801, which dramatically blocked the rise in intracellular Ca\(^{2+}\) caused by glutamate, bis(7)-tacrine also significantly inhibited the intracellular Ca\(^{2+}\) triggered by glutamate (Fig. 7). There was no significant difference in both magnitude and kinetics of the resting intracellular Ca\(^{2+}\) levels (i.e. before the addition of glutamate) between the neurons treated with the vehicle and bis(7)-tacrine (Fig. 7B).

Bis(7)-tacrine Blocks NMDA Receptors at the MK-801-binding Site—Before determining whether bis(7)-tacrine reduced glutamate-increased intracellular Ca\(^{2+}\) by directly interfering NMDA receptors, we first showed that glutamate-mediated apoptosis was completely blocked by MK-801 at 1 μM (a non-competitive antagonist of NMDA receptors) or 2-amino-5-phosphonovalerate (APV) at 50 μM (a competitive antagonist of NMDA receptors) but not affected by the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and kainate receptor antagonists 6,7-dinitroquinoxaline-2,3-dione (DNQX) at 200 μM or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) at 100 μM (Fig. 8A), indicating that glutamate-induced cell death was mainly caused by the stimulation of NMDA receptors (21, 22). To further dissect the possible interaction between bis(7)-tacrine and NMDA receptors, patch clamp analyses were performed in primary cultured hippocampal neurons. NMDA receptor-mediated whole cell currents were elicited using the fast application of 100 μM NMDA. NMDA receptor-mediated currents were measured at −60 mV. Under the control conditions the agonist-evoked current reached an initial peak and then decayed to a steady-state level of ~70~80% of the initial peak. The pooled data from eight neurons showed that in the presence of MK-801 (1 μM), bis(7)-tacrine (0.1 and 1 μM), tacrine (50 μM) or E2020 (50 μM), 100 μM NMDA-induced currents were 33 ± 3.1, 84.5 ± 4.6, 54.3 ± 3.6, 90.6 ± 5.9, and 98.4 ± 6.4%, respectively, of the control value (Fig. 8B), indicating that bis(7)-tacrine, but not tacrine and E2020, significantly reduced the current induced by NMDA. Furthermore, according to receptor-ligand binding assay, compared with MK-801 (IC\(_{50}\) = 0.0389 μM, K\(_D\) = 0.041 μM), bis(7)-tacrine moderately competed with \(^{3}H\)MK-801 with an IC\(_{50}\) of 0.763 μM, whereas tacrine (IC\(_{50}\) = 4.5 μM) and E2020 (IC\(_{50}\) = 184 μM) only weakly competed with \(^{3}H\)MK-801 in rat cerebellar cortical membranes (Fig. 8C).

**DISCUSSION**

AChE inhibitors, such as tacrine and huperzine A, have been shown to attenuate the apoptosis of cortical neurons induced by H\(_2\)O\(_2\) or β-amyloid peptide (10, 32). Bis(7)-tacrine is also able to prevent H\(_2\)O\(_2\)-induced neuronal apoptosis and ischemia-caused injury (4, 5). However, the exact mechanisms of its neuroprotective activities remain elusive. In this study, the model that glutamate at low concentrations caused apoptosis (not necrosis) was successfully set up in our CGN cultures as demonstrated by several specific apoptotic detecting methods (Fig. 2) (26). By using this model, we have investigated the neuroprotective activity and the mechanisms of bis(7)-tacrine. It was found that bis(7)-tacrine inhibited the glutamate-induced apoptosis in a dose-dependent manner (EC\(_{50}\) = 25 μM), and its preventive effect was significant even at 0.01 μM (p < 0.05; Fig. 1). Because bis(7)-tacrine is a novel, selective, and potent AChE inhibitor (1, 2), did bis(7)-tacrine prevent glutamate-induced apoptosis by inhibition of AChE? However, other AChE inhibitors (tacrine, E2020, and disopropylfluorophosphate) hardly showed any neuroprotective properties on neuronal apoptosis induced by glutamate in CGNs, although E2020 at 50 μM possessed similar effects on inhibiting the activity of AChE in CGNs with bis(7)-tacrine at 1 μM (Fig. 3, A–C). On the other hand, the activation of cholinergic receptors is able to prevent apoptosis induced by several apoptotic inducers, such as amyloid-β peptide in cortical neurons or low potassium in CGNs (27, 31, 33). Some selective AChE inhibitors, such as rivastigmine and galantamine, are able to directly activate cholinergic receptors and facilitate synaptic transmission in the mammalian central nervous system (34). In our study, neither atropine (an antagonist of muscarinic cholinoreceptors) nor dihydro-β-erythroidine (an antagonist of nicotinic cholinoreceptors) affected the neuroprotective properties of bis(7)-tacrine (Fig. 3D). Considering the above results, we propose that bis(7)-tacrine prevents apoptosis induced by glutamate independent of inhibiting the activity of AChE and cholinergic transmission. Although bis(7)-tacrine, as well as other AChE inhibitors, prevents apoptosis possibly independent of inhibiting the activity of AChE, it should not be excluded that they probably interfere noncholinergic functions of AChE (35, 36). There is increasing evidence that AChE might be involved in apoptosis (37). Transfection with AChE leads to an increase of apoptosis in retinal cells from chick embryos (38), and highly purified AChE proteins have been shown to be toxic to cells via the apoptotic mechanism (39), and increased expression of intraneuronal AChE is involved in apoptosis of SK-N-SH cells (40). AChE has been suggested to be neurotoxic in vivo and in vitro and accelerate assembly of amyloid peptide into Alzheimer fibrils (40–42). These noncholinergic roles of AChE could be important in the process of AD, and they might be influenced in a beneficial manner by AChE inhibitors and even will become a novel target for treating AD. The therapeutic effects are probably based on a multi-target mechanism, so these AChE inhibitors with anti-apoptotic activities might be much more efficient in the preventing and treating AD than those that only inhibit AChE, whereas their neuroprotective properties might be decided by not inhibiting the enzymatic activity of AChE but interfering AChE-mediated apoptosis. However, more studies should be performed to confirm this hypothesis.

It has been reported that the activation of ERK and p38 MAP pathways are implicated in the intracellular signal transduction pathways of glutamate-induced neuronal cell death from the independent groups (21, 22). However, the relationship between ERK and p38 pathways in the apoptosis induced by glutamate is unclear as yet. In our system, we have found that at their efficacy, both MEK1/2 inhibitors and p38 inhibitors can partially prevent the cell death induced by glutamate (Fig. 4). The results confirm the published findings that ERK and p38 pathways may be involved in glutamate-induced apoptosis in neurons. More interestingly, the co-application of U0126 and SB203580 almost blocked the cell death (Fig. 4B), indicating that these two kinase pathways might play a synergistic role in glutamate-induced apoptosis. Also, it has been found that bis(7)-tacrine inhibits the activation of ERK and p38 pathways caused by glutamate. According to the results obtained in immunoblots, bis(7)-tacrine inhibits the phosphorylation of Raf, MEK1/2, and ERK1/2. This overall inhibition of kinases in the ERK pathways suggests that bis(7)-tacrine possibly inhibits the upstream modules of the pathway rather than directly interferes this pathway. On the other hand, bis(7)-tacrine inhibits the activation of MKK3/6 and p38 caused by glutamate in CGNs but does not directly inhibit the activity of phospho-p38 in vitro, which also indicates that bis(7)-tacrine inhibits
Bis(7)-tacrine Prevents Neuronal Apoptosis

**Fig. 8. Bis(7)-tacrine blocks NMDA receptors at the MK-801 site.**

A. NMDA receptors mediated glutamate-induced apoptosis in CGNs. CGNs were preincubated with or without 2 μM MK-801, 50 μM APV, 200 μM DNQX, or 100 μM CNQX for 2 h and then exposed to 75 μM glutamate (Glu). At 24 h after glutamate challenge, cell viability was measured by MTT assay. The data, expressed as percentages of control, are the means ± S.E. of three separate experiments. **, p < 0.01 versus glutamate group (ANOVA and Dunnett’s test). B. Bis(7)-tacrine inhibits NMDA-evoked whole cell current in primary cultured hippocampal neurons. Upper panel, representative whole cell recordings with bars indicating the times at which NMDA (100 μM) alone, or NMDA with MK-801 (1 μM), bis(7)-tacrine (0.1 or 1 μM), tacrine (50 μM), and E2020 (50 μM) were primed in the same patch clamped cell in the presence of 10 μM glycine. NMDA was applied by a Y-tubing device for the duration indicated by the bars. Lower panel, the effects of bis(7)-tacrine on the NMDA-evoked whole cell current responses. According to the current recordings of the above groups, the data were expressed as the means ± S.E. of the ratios of normalized currents (n = 8 for each group). C. Inhibition of [3H]MK-801 binding to rat cerebellar cortical membranes by bis(7)-tacrine. The membranes from rat cerebellar cortex were incubated with [3H]MK-801 (4 nM) and the chemicals indicated at gradually increasing concentrations as described under “Experimental Procedures.” The data, expressed as percentages of control, were the means according to three experiments, and the graphs were plotted by Sigmaplot software as described under “Experimental Procedures.”

FIG. 8. Bis(7)-tacrine blocks NMDA receptors at the MK-801 site. A. NMDA receptors mediated glutamate-induced apoptosis in CGNs. CGNs were preincubated with or without 2 μM MK-801, 50 μM APV, 200 μM DNQX, or 100 μM CNQX for 2 h and then exposed to 75 μM glutamate (Glu). At 24 h after glutamate challenge, cell viability was measured by MTT assay. The data, expressed as percentages of control, were the means ± S.E. of three separate experiments. **, p < 0.01 versus glutamate group (ANOVA and Dunnett’s test). B. Bis(7)-tacrine inhibits NMDA-evoked whole cell current in primary cultured hippocampal neurons. Upper panel, representative whole cell recordings with bars indicating the times at which NMDA (100 μM) alone, or NMDA with MK-801 (1 μM), bis(7)-tacrine (0.1 or 1 μM), tacrine (50 μM), and E2020 (50 μM) were primed in the same patch clamped cell in the presence of 10 μM glycine. NMDA was applied by a Y-tubing device for the duration indicated by the bars. Lower panel, the effects of bis(7)-tacrine on the NMDA-evoked whole cell current responses. According to the current recordings of the above groups, the data were expressed as the means ± S.E. of the ratios of normalized currents (n = 8 for each group). C. Inhibition of [3H]MK-801 binding to rat cerebellar cortical membranes by bis(7)-tacrine. The membranes from rat cerebellar cortex were incubated with [3H]MK-801 (4 nM) and the chemicals indicated at gradually increasing concentrations as described under “Experimental Procedures.” The data, expressed as percentages of control, were the means according to three experiments, and the graphs were plotted by Sigmaplot software as described under “Experimental Procedures.”

Previous literature has reported that the rise of intracellular Ca²⁺ level can activate both ERK and p38 pathways under many conditions (21, 22). On the other hand, the overloading of intracellular calcium is necessary for glutamate-induced cell death in CGNs (18). The patterns of glutamate-stimulated calcium in neuronal cells are very complicated, mainly involving calcium influx through the NMDA receptor ion channel and intracellular calcium mobilization (43). Blockade of intracellular calcium release or the presence of chelators of intracellular calcium is neuroprotective against glutamate-mediated cell death (44). We have demonstrated in another study that calcium chelators, such as EDTA at 2 mM and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N''-tetraacetic acid-acetoxymethylester at 0.5 μM, are able to prevent the cell death in CGNs caused by glutamate (data not shown). It may be possible that stabilization of intracellular Ca²⁺ is an effect produced by bis(7)-tacrine. The confocal microscopy techniques showed that bis(7)-tacrine reversed the rise of the intracellular calcium caused by glutamate to the nearly basal level. In addition, MK-801 and APV prevent neuronal cell death induced by glutamate because they can decrease calcium influx via directly blocking the NMDA receptor (21, 22). Our results also point to the direction that the action of bis(7)-tacrine may be further upstream rather than directly on intracellular Ca²⁺ release. In the whole cell patch clamp assay, bis(7)-tacrine significantly reduced the currents in neurons evoked by 100 μM NMDA, whereas E2020 and tacrine at about several times higher than therapeutic concentrations could not yet (Fig. 8B). More interestingly, bis(7)-tacrine can moderately compete with [3H]MK-801 in rat cerebellar cortical membranes. The results indicate that the inhibition of bis(7)-tacrine to glutamate-induced apoptosis may be partially fully contributed by directly blocking NMDA receptors at the MK-801 site. Increasing evidence shows that glutamate-induced cytotoxicity is involved in AD, and moderately blocking NMDA receptors may be more helpful for treating AD (45). The strongest evidence is that memantine, which is a moderate antagonist of NMDA receptors at the MK-801 site and able to prevent glutamate-induced apoptosis in neurons, has been approved by the United States Food and Drug Administration for treating AD patients, whereas early drugs for treating AD were only limited in AChE inhibitors (13, 46). Compared with the Kᵢ values of memantine competed with [3H]MK-801 (Kᵢ = 0.54–1 μM; 46), the Kᵢ value of bis(7)-tacrine is 0.695 μM on cerebellar cortical membranes, indicating that bis(7)-tacrine and memantine have a similar affinity to NMDA receptors. This narrow range of moderate affinity to NMDA receptors has actually high significance in the treatment of the p38 pathway by interfering with the upstream modules of this pathway. Taking the evidence together, bis(7)-tacrine inhibits glutamate-induced apoptosis most likely via the inhibition of the common upstream modules of ERK and p38 pathways, which may explain why bis(7)-tacrine possesses more potent neuroprotective effects than the inhibitors of ERK pathway or the inhibitors of p38 alone.
numerous central nervous system disorders because the affinity is associated with blocking kinetics to NMDA receptors (13, 46). High affinity antagonists, such as MK-801, are associated with very slow kinetics and weak voltage dependence, and produce highly undesirable side effects at doses within their putative therapeutic range, whereas very low affinity antagonists, such as magnesium ion, show fast blocking kinetics and strong voltage dependence and may have no therapeutic potential on their tolerated doses (13). A number of clinical trials have demonstrated that treatment with memantine provides significant functional benefits for patients with AD patients essentially devoid of such side effects (47). More encouragingly, well controlled trials of memantine/E2020 dual therapy have shown superior efficacy in moderate to severe AD patient subgroups (48). Therefore, we conjecture that bis(7)-tacrine, a potent AChE inhibitor and moderate antagonist of NMDA receptors, might slow pathogenesis in addition to improving memory and cognition in AD patients (13, 49).

In conclusion, we demonstrate that bis(7)-tacrine inhibits glutamate-mediated apoptosis in neurons through the blockade of NMDA receptors at the MK-801-binding site. This function appears to be independent of inhibiting AChE and cholinergic transmission. Moreover, glutamate induces neuronal apoptosis by activating both the ERK and p38 pathways that play a synergistic role in the downstream apoptotic signals of overloading intracellular Ca$^{2+}$. Bis(7)-tacrine buffers the rise of intracellular Ca$^{2+}$ caused by glutamate (Fig. 7) through blocking NMDA receptors (Fig. 8) and then inhibits two MAPK pathways: ERK and p38 pathways (Figs. 5 and 6). Combining these and our previously published results (1–5), it is conjectured that bis(7)-tacrine concurrently possesses anti-NMDA and anti-AChE activities. This synergism might most effectively prevent the neurodegeneration in addition to alleviating the cognitive impairment for AD patients from early to late stages.

Acknowledgments—We sincerely thank Dr. Zhenguo Wu for kindly providing materials and equipments used in the experiments. We also express our appreciation to Ling Nga Chan for assistance in analyzing the data of whole cell patch clamp, to Wai Chung Leung for help in setting up the receptor-ligand binding assay, and to Dr. Kelvin K. W. Kan for proofing our manuscript.

REFERENCES